

## Characteristics of the Constituent Substrains of *Bacillus popilliae* Growing in Batch and Continuous Cultures

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Continuous culture of *Bacillus popilliae* was achieved for the first time in a small chemostat. Initially, variable cell yields during steady-state chemostat growth led to a re-examination of growth rates in batch cultures. *B. popilliae* NRRL B-2309 and a wild strain were both found to be natural mixtures of three substrains characterized by different growth rates and colony morphologies and varying stability. Selected subcultures grown continuously provided data for three different cell production curves. Cell yields were two to three times greater per unit of medium in continuous than in batch culture, and about 1% of slow-growing chemostat cells formed typical spores.

Large-scale fermentative production of infective spores of *Bacillus popilliae* Dutky would supply a proven biological insecticide (3, 14) to control the Japanese beetle, *Popillia japonica* Newman. Suitable vegetative proliferation of this entomopathogen has been reported (9), and methods for obtaining limited sporulation in liquid and on solid medium have been described (5, 10, 15). Nevertheless, a means to induce extensive sporulation of *B. popilliae* growing in laboratory media has evaded discovery.

There are indications, however, that slow, steady vegetative proliferation is a prerequisite to spore formation. Vigorous growth has not been associated with the subsequent formation of spores either in vitro or in vivo. For example, *B. popilliae* did not form spores in shake-flask cultures of rich medium in which the average cell generation time or division time ( $t_d$ ) was about 3 h or in a dialysis system when  $t_d$  was reduced to 2 h, the shortest time we have observed (9). Limited sporulation has occurred in flask cultures containing activated carbon (5) where  $t_d$  was about 3.5 h. In colonies where  $t_d$  was between 9 and 11 h, the incidence of sporulation was about 20% (10). Generation time of the sporulating cultures of Wyss (15) was similarly extended. *B. popilliae* grew very slowly in the hemocoel of Japanese beetle larvae, requiring 18 to 19 h for cell division; large numbers of spores accumulated (8) in larval hemolymph.

We do not contend that division time alone is responsible for sporulation, because special laboratory-developed strains of *B. popilliae* and

select yeast extracts were required to produce spores in liquid medium with carbon and also in colonies on solid medium (5, 10). Nevertheless, the evidence strongly suggests that slower growth rates should be investigated as a means for obtaining sporogenic cells.

We have decreased growth rate in batch cultures ( $t_d = 4$  to 5 h) by using less nutritious media and by limiting other conditions, but cell yields were very low. In continuous cultivation, growth rate is determined by dilution rate. Continuous cultures can be maintained at extremely slow growth rates by restricting the input of fresh medium. The method is especially appropriate for our purpose, since certain other aspects of continuous cultivation are similar to the growth situation of *B. popilliae* in Japanese beetle larvae. Therefore, we have developed a chemostat for the cultivation of *B. popilliae*. Variable cell yields of different *B. popilliae* cultures at identical steady-state conditions led to a re-evaluation of batch culture growth rates and colonial morphology. The natural substrains found in *B. popilliae* were characterized by colony type, and certain correlations between colony type, growth rate, the persistence of viability, and infectivity have been established. We believe that the characteristic of variability as applied to *B. popilliae* (2, 6, 7, 10-12, 15) is created by the constituent substrains. Practical application of the knowledge of variability in *B. popilliae* cultures may be imminent. Japanese beetle populations are increasing in areas where previously they had been controlled for years by milky disease. Spores collected from diseased larvae in these areas are neither as infective as noted previously nor as infective as spores that

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have been stored for several years. Dunbar and Beard (1) conducted the infectivity tests and concluded that *B. popilliae* is attenuating.

## MATERIALS AND METHODS

**Organism and culture media.** *B. popilliae* Dutky, strain NRRL B-2309 or substrains derived therefrom, was used in chemostat, flask, and colony studies. B-2309 is a basic strain from our collection (4) of *B. popilliae*. It causes typical type A milky disease in Japanese beetle larvae. Vegetative cells remain viable after lyophilization, affording an unlimited supply of homogeneous inoculum.

Maximum vegetative populations of *B. popilliae* have been obtained in batch culture by using a rich medium containing 1.5% Difco yeast extract, 0.2% glucose, and 0.3%  $K_2HPO_4$  (milky disease [MD] medium [9]). Since yeast extract alone will support significant growth, it has been impossible to formulate a medium in which glucose is truly limiting or in which yeast extract concentration can be varied without affecting growth. Synthetic media have been described by Sylvester and Costilow (13) and by Wyss (15), but neither medium supported vegetative populations necessary for continuous growth. Our medium for chemostat culture, designated CC medium, was a compromise that provided suitable chemostat growth. It contained 0.5% yeast extract, 0.1% glucose, 0.3%  $K_2HPO_4$ , and "G" salts. To prevent precipitation and facilitate filter sterilization, reduced concentrations of salts were added as follows: 0.05%  $(NH_4)_2SO_4$ , 0.002%  $MgSO_4$ , 0.0008%  $CaCl_2 \cdot 2H_2O$ , and 0.0005%  $MnSO_4 \cdot H_2O$ . Growth was controlled by variations in feed rate of this total medium and not by independent variation of glucose or yeast extract concentration; therefore, the specific growth limitation is unknown.

Colonial studies used a rich medium containing 1.5% Difco yeast extract, 0.2% glucose (autoclaved sepa-

ately), 0.3%  $K_2HPO_4$ , and 0.2% Difco agar (MD agar medium [9]).

Total counts of batch cultures, colonies, and steady-state cells taken from the chemostat were made using a phase-contrast microscope and a Petroff-Hausser bacteria counting chamber. Viable counts were determined by standard dilution and plating techniques. A chemostat sample of 0.5 ml was sufficient for cell counts and pH. A period of 3 h between samplings minimized perturbation of steady-state conditions.

**Chemostat operation.** The chemostat was a 100-ml, jacketed, Bellco spinner flask, modified to have five ports (Fig. 1). The small size was convenient to handle during autoclaving. Also, 8 liters of medium was sufficient to operate the small chemostat for over 16 days at a dilution rate of  $2\ h^{-1}$  or for 2 months at a  $0.05\ h^{-1}$  dilution rate. Fresh medium was metered into the chemostat by a Sigmamotor "kinetic clamp" variable flow pump, model AL 2E. When equipped with Tygon or silicone rubber tubing (1/16-inch [ca. 1.59-mm] ID by 1/32-inch [ca. 0.79-mm] wall), delivery of this pump ranged from  $<2$  to  $>30$  ml/h. A small in-line buret was used periodically to determine the precise flow rate. After an initial period allowing for deformation of the tubing, flow rate stabilized for any constant pump speed. Stirring was accomplished by a Bellco nonheating magnetic stirrer. Filter-sterilized air was introduced through a small porous sparger. Aeration at 1 volume of air per volume of medium per min was not limiting at the highest dilution rates studied. Temperature was maintained at  $28 \pm 1^\circ C$  by circulation of water through the chemostat jacket.

Excess culture was forced from the chemostat by internal air pressure whenever the liquid level rose to cover the outlet. Overflow was collected in a sterile ice-cooled flask or in a waste reservoir. Foam resulting from sparging was a problem because it entered the outlet tube and lowered the liquid level by an amount equal to the height of foam. Sufficient antifoam to

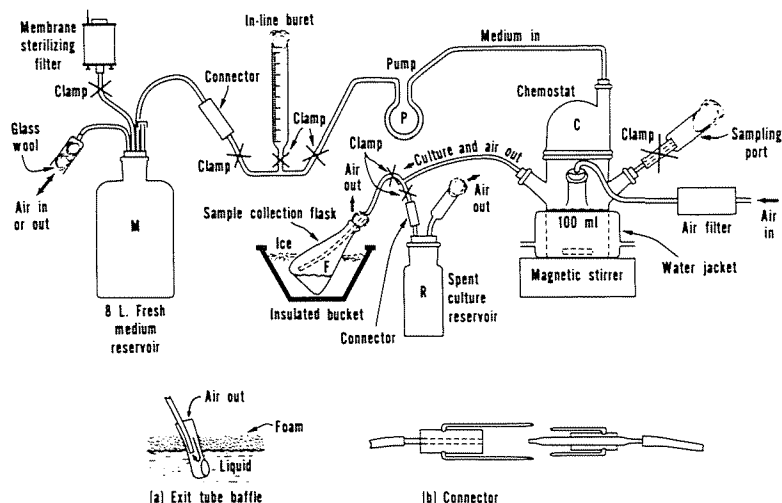


FIG. 1. Chemostat assembly. Filter-sterilized medium (M) is metered into the chemostat (C) by pump (P). Outflow is collected in ice-cooled flask (F) or shunted to reservoir (R). Details of exit tube baffle and slip connector shown in (a) and (b).

completely control foaming inhibited growth. We compromised by adding only 25 ml of sterile 5% (vol/vol) emulsion of Hodag FD-62 to 8 liters of filter-sterilized medium. Foam was depressed to 6 or 8 mm in thickness. A baffled exit tube (Fig. 1a) prevented rapid exit of the foam and maintained a constant liquid level when foam thickness varied.

Preferably, the chemostat and all fittings, including the sterilizing filter, and all medium and waste reservoirs should be assembled and sterilized simultaneously by autoclaving. This procedure was eliminated at some risk by the use of simple connectors (Fig. 1b), which permitted the coupling of presterilized subassemblies. Such fittings also facilitated the changing of reservoirs during an experiment, although the risk of contamination was increased whenever tubes containing medium or culture (wet lines) were disconnected. Wet lines were clamped near the point of disconnection to prevent movement of liquid that would draw air into the lines.

Inocula for the chemostat consisted of 10 ml of B-2309 culture grown in a shaken flask of MD medium. Inocula were transferred during midexponential phase of growth (10 to 12 h). About 6 h of growth (batchwise) in the chemostat should precede input of fresh medium.

## RESULTS

**Batch culture.** Problems incurred in attempting to establish young cultures in the chemostat and their erratic specific growth rates prompted a closer quantitative study of the growth of the B-2309 strain in flasks and resulted in the following information.

We have known that B-2309 cultures, when started from either lyophilized cells or germinated spores, do not grow well at first, producing only  $2 \times 10^8$  or  $3 \times 10^8$  cells per ml in shaken flasks of MD medium. They were easily lost, especially if the transfer interval was increased beyond 24 h. After four or five transfers in liquid medium, cell production usually increased to  $6 \times 10^8$  or  $7 \times 10^8$  cells per ml, and about 50% of these cultures remained at this second growth level for many additional serial transfers (Table 1, item j), but a few cultures increased to about  $1 \times 10^9$  cells per ml. This latter level of growth rate was more stable, and most cultures remained in this category (Table 1, item f). Only a few cultures reached a higher and apparently final level of cell production of over  $2 \times 10^9$  viable cells per ml of MD medium in shaken flasks in 24 h.

Older cultures have always grown more rapidly than young cultures, but the increase in growth rate was thought to occur gradually, requiring many transfers. Now it appears that increases in growth rate can occur within a few transfers in liquid medium, especially if the transfer intervals are less than 24 h.

Normally, cultures were transferred at ap-

proximately 24-h intervals using 2% inoculum, 50 ml of MD medium in 250-ml Erlenmeyer flasks, and rotary stirring at 28°C.

**Chemostat culture.** Our first successful chemostat experiments used a vigorous culture of B-2309 that had been maintained for several months by daily transfer in shaken flasks of MD medium in which it produced over  $2 \times 10^9$  cells per ml (Table 1, item a). At first it was considered to be a normal fast-growing B-2309 culture; however, evidence presented herein indicates that it is a pure new substrain and is designated hereafter as NRRL B-4265, *B. popilliae*. It was used in flask culture to show that filter sterilization of the CC medium encouraged 30% more growth than autoclaving (Table 1, compare items b and c).

The B-4265 culture grew very well in the chemostat, as depicted by curve 1 of Fig. 2, which shows cell productivity at steady states established at a range of dilution rates. Cell production in the chemostat was two to three times that in batch cultures of the same CC medium (compare items b and c with items d and e, Table 1). Batch cultures of *B. popilliae* will not grow at pH below 6.5, but steady-state growth of B-4265 in the chemostat was observed at pH 6.2, although normally pH of chemostat cultures was 6.5 to 6.8.

During attempts to establish a washout point, the B-4265 culture was temporarily lost, and fresh cultures were started from lyophilized stored cells of B-2309.

As mentioned previously, when the young B-2309 batch cultures were grown in the chemostat, steady-state cell yields indicated that their specific growth rates were variable. Cultures fresh from lyophil could not be established in a continuous growth situation, but "second-level" batch cultures ( $6 \times 10^8$  cells per ml) were grown in CC medium in the chemostat (Table 1, item k). Curve 3 of Fig. 2 represents steady-state cell yields of such cultures at dilution rates from 0.025 to  $0.16 \text{ h}^{-1}$ . These cultures established new stable steady states and a cell yield curve different from that of B-4265. At dilution rates near  $0.14 \text{ h}^{-1}$  and above, it became impossible to reach a new steady state on the established curve. Cell yields became variable at  $D = 0.14 \text{ h}^{-1}$ , but they occasionally stabilized in a new steady state on the next higher curve (curve 2, Fig. 2), indicating an abnormal increase in specific growth rate. Steady state was established only after the increase in specific growth rate occurred. A shift to lower specific growth rate was not observed.

Curve 2 of Fig. 2 represents cell yield from chemostat cultures of B-2309 in the "third level"; i.e., they had produced  $1 \times 10^9$  cells per ml in

TABLE 1. Cell production by *B. popilliae* NRRL B-2039 and substrains in batch and continuous culture

Culture identification		Medium	Sterilization procedure	Culture system	D or other information	Microscopic cell counts per ml
NRRL	Previous growth					
(a) Substrain B-4265	From B-2309, grown from lyophil and carried in MD medium for >100 transfers	MD	Steam <sup>a</sup>	Shaken flask	2% inoculum from flask	$2.2 \times 10^8$ at 24 h
(b) Substrain B-4265	From B-2309, grown from lyophil and carried in MD medium for >100 transfers	CC	Steam	Shaken flask	2% inoculum from flask	$575 \times 10^6$ at 24 h
(c) Substrain B-4265	From B-2309, grown from lyophil and carried in MD medium for >100 transfers	CC	Filter sterilized	Shaken flask	2% inoculum from flask	$745 \times 10^6$ at 24 h
(d) Substrain B-4265	From B-2309, grown from lyophil and carried in MD medium for >100 transfers	CC	Filter sterilized	Chemostat	$D = 0.09$ to $0.1 \text{ h}^{-1}$	$1.4 \times 10^8$ at steady state
(e) Substrain B-4265	From B-2309, grown from lyophil and carried in MD medium for >100 transfers	CC	Filter sterilized	Chemostat	$D = 0.16 \text{ h}^{-1}$	$1.8 \times 10^8$ at steady state
(f) B-2309, new culture	From lyophil, carried in MD medium for 33 transfers	MD	Steam	Shaken flask	2% inoculum from flask	$1.2 \times 10^9$ at 24 h
(g) B-2309, new culture	From lyophil, carried in MD medium for 33 transfers	CC	Filter sterilized	Shaken flask	2% inoculum from flask	$550 \times 10^6$ at 24 h
(h) B-2309, new culture	Same as above after 1 week in chemostat	CC	Filter sterilized	Chemostat	$D = 0.09$ to $0.1 \text{ h}^{-1}$	$500 \times 10^6$ at steady state
(i) B-2309, new culture	Same as above after 3 weeks in chemostat	CC	Filter sterilized	Chemostat	$D = 0.14$ to $0.20 \text{ h}^{-1}$	$700 \times 10^6$ at steady state
(j) B-2309, second new culture	From lyophil, carried in MD for 12 transfers	MD	Steam	Shaken flask	2% inoculum from flask	$650 \times 10^6$ at 24 h
(k) B-2309, second new culture	From lyophil, carried in MD for 12 transfers	CC	Filter sterilized	Chemostat	$D = 0.075 \text{ h}^{-1}$ $D = 0.12 \text{ h}^{-1}$	$160 \times 10^6$ at steady state $200 \times 10^6$ at steady state
(l) B-2309, second new culture	From lyophil, carried in MD for 12 transfers	CC	Filter sterilized	Shaken flask	2% inoculum from chemostat	$160 \times 10^6$ at 24 h

<sup>a</sup> Steam-sterilized glucose and salts were both sterilized separately and added aseptically before inoculation.<sup>b</sup> Antifoam, when used, was steam sterilized separately and added aseptically to the filter-sterilized whole medium.

shaken flasks (Table 1, item f). Maximum cell production of such cultures at steady states in the chemostat occurred between  $D = 0.14 \text{ h}^{-1}$  and  $D = 0.20 \text{ h}^{-1}$ , the yield being  $7 \times 10^8$  cells per ml of CC medium (Table 1, items h and i). The washout point was below a dilution rate of  $0.29 \text{ h}^{-1}$ , at which point population decline during 8 and 24 h was measured (Fig. 2). The

maximum specific growth rate was calculated from this data; it equaled 0.252, corresponding to  $t_d = 2.7 \text{ h}$ . Steady state was established only once at  $D = 0.235 \text{ h}^{-1}$ , corresponding to  $t_d$  of 2.95 h.

Refractile spores of *B. popilliae* were only formed in cultures that established curves 2 and 3 of Fig. 2. The incidence of sporogenesis was

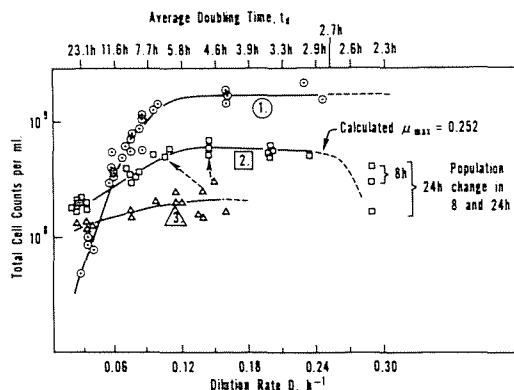


FIG. 2. Steady-state cell yields of *B. popilliae* cultures growing in the chemostat. Data for curve 1 are entirely from substrain NRRL B-4265. Data for curve 2 are from pure cultures containing only one colony type and cultures containing essentially one colony type but with a few cells that produce both faster- and slower-growing colonies. Data for curve 3 are from younger, slower-growing cultures containing cells that produced all three colony types.

about 1% and occurred at dilution rates below  $0.1 \text{ h}^{-1}$ . Sporulation was not improved in chemostat cultures of the vegetative progeny of these spores. Sudden downshift from higher specific growth rate to dilution rates below  $0.1 \text{ h}^{-1}$  was usually followed by sporogenesis, but at the same low level.

**Colony culture.** The distinct morphological characteristics and differences in cell content of mature colonies on viable count plates from batch and continuous cultures was the first indication that growth rates were correlated with colony types. Strain B-2309, isolated from a commercial spore dust, was lyophilized (4) after a minimum of vegetative transfers. On MD agar medium, revived cultures produced three different types of colonies; all were circular with slightly undulate margins. The most numerous type (70%) resembled a fried egg with amber pigment in the raised center, a clear surrounding growth, and umbonate elevation. Less numerous (20%) was a regular colony type previously associated with *B. popilliae* (10). These regular colonies were largest, with only slight pigmentation and simple convex elevation. A few (10%) small colonies, always present, were of pulvinate elevation with heavy pigmentation and were called amber beads. The three colony types are shown in Fig. 3.

After dilution and replating, cells from the three colony types produced new colonies approximately as shown in Fig. 4. Each colony in these experiments represented growth from a single inoculum cell, but the cells did not always

reproduce their previous colony type. On (rich) MD agar medium, a spontaneous progression from amber bead to fried egg to regular colonies occurred, with a concurrent increase in stability. Succeeding colony types were larger, and they contained more cells. At the same age, regular-type colonies contained 10 and 100 times more cells than fried egg and amber bead colonies, respectively. Since colonial growth is exponential only in early stages, colony counts could not be used to establish growth rate. The progressions and reversions that occurred from plate to plate presumably occur also from liquid to plate, obscuring the true ratio of colony types in liquid cultures. When the three colony types were plated on solidified CC medium in an attempt to limit progression by limiting growth rate, fried egg and regular colonies were repressed in numbers, and amber beads did not appear at all. Fewer fried egg colonies on CC agar suggested that reversion had occurred; if amber bead colonies were present, they did not develop to visible proportions. Presumably, amber bead colonies require a richer medium for development.

The different colony types are not caused by the stress of lyophilization or storage of cells, because all three colony types arise from germinated B-2309 spores. Incidentally, they therefore should all represent sporogenic strains.

As growth rate increased in liquid cultures after several transfers, amber bead colonies disappeared, but fried egg colonies persisted in platings from all but the most rapidly growing cultures. Cells that produced fried egg colonies had greater longevity than cells that produced the other two colony types. Flask cultures that contained three colony types when plated at 24 h produced only fried egg colonies when plated after an additional 5 days of still culture. Total viable population of such flasks was about  $20 \times 10^6$  cells per ml at the time of plating (Fig. 5a). Continued shaking caused death of all cells within about 3 days.

On rich agar medium, cells had a strong tendency to progress from amber bead to fried egg and finally to regular colonies, but there was also a weak tendency in the reverse direction which functioned with longevity or persistence to preserve slower-growing colony types. In spite of this, a few cultures of single colony type developed after extensive transfer in batch culture. The B-4265 culture which produced only regular-type colonies (Fig. 5b) developed in this manner. Two young cultures, originally showing three colony types, were reduced to pure fried egg colonies after prolonged chemostat cultivation at intermediate and lower growth rates (Fig.

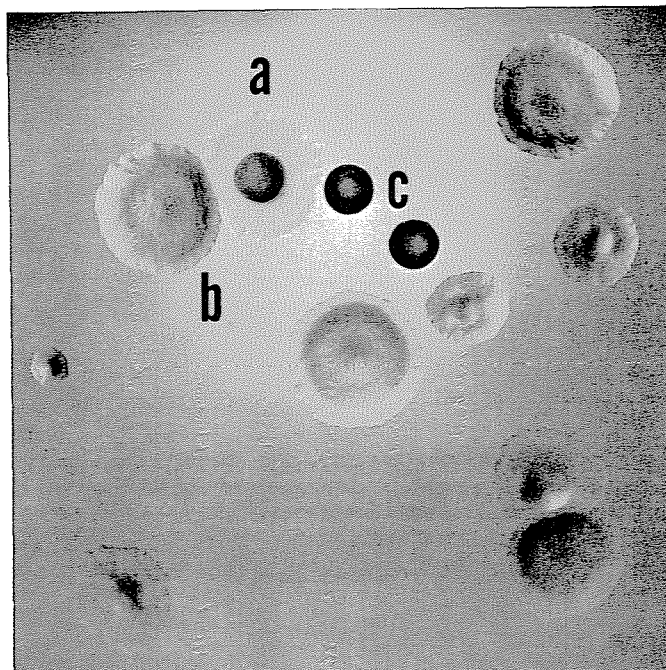


FIG. 3. The three colony types of *B. popilliae* B-2309. Fried egg (a), regular (b), and amber bead (c).

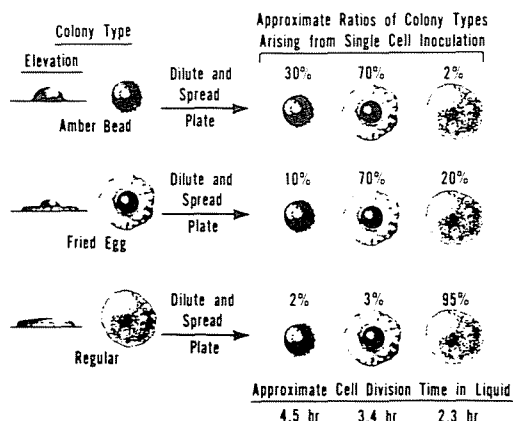


FIG. 4. Progression and reversion of colony types when inoculum from each is diluted and replated on rich MD agar medium.

5c). The cell yield data of these two cultures fit curve 2, Fig. 2. Inoculum from two 5-day still-culture flasks (Fig. 5a) also gave cell yields in the chemostat that fit curve 2, Fig. 2.

After lyophilization and a storage period, single colony-type cultures demonstrated the same growth characteristics and colony type after they were revived. Progression and reversion did not occur; once pure, the different cell or colony types were stable. When multiple-cell inoculum was transferred directly from colony to fresh

plate by wire loop, the resultant colonies were quite different from colonies produced by single cells, as shown in Fig. 6. Inoculum from either the periphery or the center "yolk" of fried-egg colonies yielded one colony type (Fig. 6a), with rough, pitted, amber-colored centers, a smooth surrounding band of amber-colored growth, and a slight bordering band of clear growth. Cells from amber bead colonies yielded colonies as shown in Fig. 6b. They are of amber color, rough, and pitted overall. Regular colonies yielded the same regular colonies by this inoculation method (Fig. 6c).

## DISCUSSION

Our studies reveal that *B. popilliae*, whether originating from lyophilized vegetative cells (4) in our collection or from spores produced in larvae infected in the field, is a mixture of three different substrains. These exhibit different colonial morphologies and growth rates and varying stability. Limited correlation with asporogenesis and pathogenicity are also noted. Certain unusual (batch) growth characteristics of *B. popilliae*, such as the lack of a stationary phase, sensitivity to acid pH, and a lengthy cell division time, do not prevent continuous culture. Before we realized that growth rate of *B. popilliae* cultures could increase stepwise and yet be relatively stable at different levels, our cell yield data at various steady states in the chemostat

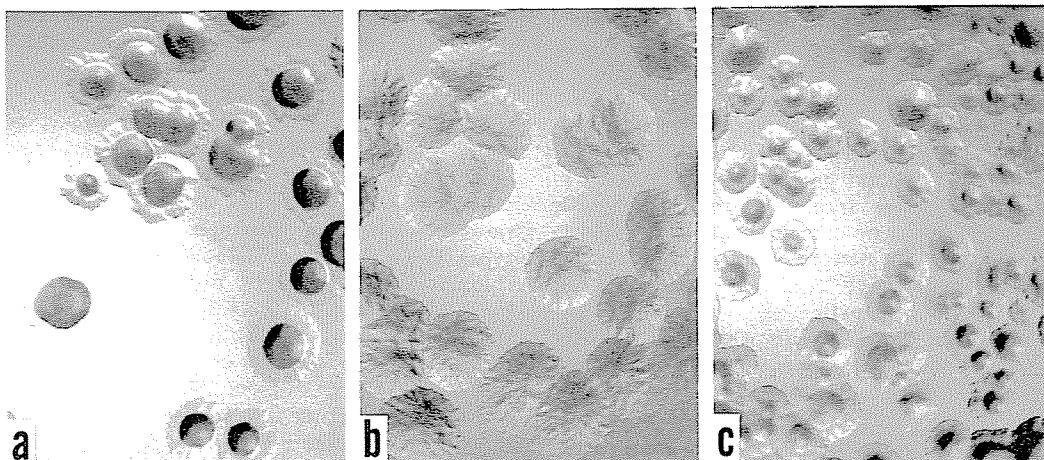


FIG. 5. Pure, stable single colony-type cultures. (a) Only cells that produced fried egg colonies persisted in a shaken flask of mixed colony types after 5 days of still culture; (b) B-4265, a rapidly growing culture; (c) a pure fried egg culture produced in the chemostat by prolonged growth at low and intermediate growth rates.

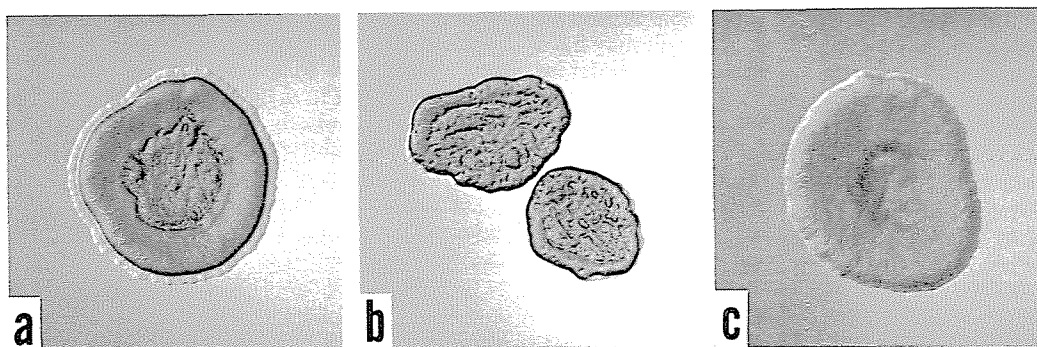


FIG. 6. Colonies produced by loop (multiple-cell) transfer. (a) Inoculum from fried egg colony only or mixed with cells from a regular colony; (b) inoculum from amber bead colony; (c) inoculum from regular colony.

did not provide a simple yield curve. Once aware of this unusual characteristic, it became evident that our early data roughly defined three different curves and not a single curve. Subsequent chemostat experiments with new cultures, carefully identified as to growth rate, verify curves 1 and 2 of Fig. 2. Cultures with cell productivity typical of curve 3, Fig. 2, were unstable and were not studied extensively.

During batchwise operation following inoculation of the chemostat, faster-growing cells tend to become dominant, as indicated by colony studies. The dominance of fast-growing cell types is preserved during steady state. Proportional changes in populations occur again during the next transient state following a change in dilution rate, but cells that produce the three colony types are usually preserved. At low dilution rates, however, slower-growing cells (fried egg colony type) achieved dominance for some

unknown reason. Above  $D = 0.13$  to  $0.14 \text{ h}^{-1}$ , the slowest-growing cells (amber bead colonies) were slowly washed out because of growth disadvantage (estimated  $t_d = 4.5$  to  $5 \text{ h}$ ). Note in curve 3, Fig. 2, that cultures became unstable at this point, tending to a more rapid specific growth rate. A few amber bead colonies were observed in chemostat samples plated at this time, but they may have resulted from reversion.

Cells of intermediate growth rate (fried egg colonies) began to wash out at  $D = 0.24 \text{ h}^{-1}$  ( $t_d = 2.9 \text{ h}$ ), and pure fast-growing cells identified as regular colony cells were produced by prolonged growth at  $D = 0.25 \text{ h}^{-1}$ . Such cultures were precariously near their washout point. Pure fast-growing cells of a single regular colony type have the highest specific growth rates at  $D > 0.06$  to  $0.07 \text{ h}^{-1}$ , but they have the lowest apparent specific growth rates at lower dilution rates. This is probably a manifestation of their increased



death rate at low dilution rates, at which such cells are less than 50% viable. Experiments with batch cultures indicate that *B. popilliae* does not have a stationary phase; viability declines at a rapid rate following exponential growth (10). However, when glucose was added 4 h after the end of exponential growth, it was utilized at about the same rate as during exponential growth, causing no change in the declining viability curve. The nonproliferating cells were still metabolically active. Yeast extract is not limiting at this stage of growth.

Sporulation of *B. popilliae* strain B-2309 was observed in the chemostat at low dilution rates. The 1% incidence of spore formation occurred first after 7 days of slow growth. Sporulation was unusual and novel in that neither the cultures nor the yeast extract were selected for sporulation beforehand. Inoculum-containing spores from the chemostat grew in shaken flasks of CC medium, but did not form spores. Slight but visible growth on the chemostat walls complicates any assessment of the accomplishment, since the colony-like proximity of cells may have contributed to sporogenesis. However, colonies of this same culture did not form spores on solidified CC, MD, or MYPT medium (10).

Cell production in flasks and in the chemostat could not be correlated precisely with colony type except in experiments using pure colony-type cultures. Other cultures were always mixtures of two or three colony types, and when they were proliferating together, progression and reversion in the cultures and during growth on solid medium precluded an accurate measurement of different colony-type cells. While viable counts and total counts of cells produced at steady states in the chemostat were not equal, they did not vary significantly for pure colony cultures or for mixed colony-type cultures. Mixed cultures in the chemostat were predominantly one colony type, as indicated by plate studies.

The first two stepwise increases in cell production in batch cultures are thought to be caused by proportional changes in cell populations. The disappearance of amber bead cells and then fried egg cells is concurrent with the final two steps. Progression and reversion tendencies diminish and are finally lost in pure, single colony-type cultures. Regular and fried egg cells were isolated and grown both in batch and continuous culture. A pure culture of amber bead-type cells has never been obtained. The  $t_d$  of the pure fried egg-type culture in flasks was about 3 h, and it produced  $1 \times 10^9$  cells per ml in MD medium. Other cultures, mixtures of fried egg- and regular colony-type cells, grew at about the same rate, but cultures containing all three col-

ony types normally produced only  $6 \times 10^8$  to  $7 \times 10^8$  cells per ml. The pure regular colony-type culture, B-4265, had a  $t_d$  of about 2.3 h in flasks of MD medium.

Colonies initiated by loop transfer from other colonies showed differences in morphology that are difficult to explain. They may result from competition between cells of different growth rates attempting to produce different colony types. Regular colony-type cells produce regular colonies (Fig. 6c) by both inoculation methods, but a mixture of regular colony cells and fried egg colony cells generates colonies identical to those from pure fried egg colony cells (Fig. 6a). We are unable to explain the different colony characteristics other than to say that they are due to growth-rate differences; the vegetative cells of all three colonies appear to be microscopically identical in appearance and motility.

Recently, we have determined that vegetative cells of the fast-growing culture, NRRL B-4265, are not infective when injected into the hemocoel of Japanese beetle larvae. This does not mean that all fast-growing cultures are noninfective. Vegetative cells or spores of NRRL B-2309M were infective by injection, but grew rapidly in larvae, usually causing death before extensive sporulation was completed (10). The normal development of milky disease caused by B-2309 requires a lengthy period, during which microscopic counts of cells in larval hemolymph indicate that their  $t_d$  is 18 to 19 h. Therefore, it is possible that a selection of cells occurs in vivo and that slower-growing cells constitute a major portion of the in vivo population ultimately responsible for sporulation. Sporulation in the chemostat occurred in cultures which were mixtures of cells of slow and intermediate growth rate.

There have been numerous reports of the continuous culture of mixtures of microorganisms, each with different metabolic and nutrient requirements and widely different growth rates. Usually in such mixed cultures, one of the microorganisms is dependent (for nutrient) on a metabolic product of the other. They are thus forced to grow at about the same rate. We believe that ours is the first report of the continuous culture of a mixture of substrains of one organism in which growth rate varies, but nutrient requirement and metabolism are mutually about the same.

Hopefully, the information we have accumulated will be helpful to others interested in the selection of varieties from the same growth population, since some of the implications involve development, differentiation, and aging of cell populations of all kinds, including those found in man.



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